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Role of Positional Hydrophobicity in the Leishmanicidal Activity of Magainin 2

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The emergence of membrane-active antimicrobial peptides as new alternatives against pathogens with multiantibiotic resistance requires the design of better analogues. Among the different physicochemical parameters involved in the optimization of linear antimicrobial peptides, positional hydrophobicity has recently been incorporated. This takes into consideration the concept of the topological distribution of hydrophobic residues throughout the sequence rather than the classical concept of hydrophobicity as a global parameter of the peptide, calculated as the summation of the individual hydrophobicities of the residues. In order to assess the contribution of this parameter to the leishmanicidal mechanisms of magainin 2 analogues, the activities of two of these analogues, MG-H1 (GIKKFLHIIWKFIKAFVGEIMNS) and MG-H2 (IIKKFLHSIWKFGKAFVG EIMNI), which have similar charges, amino acid compositions, and hydrophobicities but different positional hydrophobicities, against *Leishmania donovani* promastigotes were assayed (T. Tachi, R. F. Epand, R. M. Epand, and K. Matsuzaki, Biochemistry 41:10723-10731, 2002). The activities were compared with that of the parental peptide, F5W-magainin 2 (GIGKWLHSAKKFGKAFVGEIMNS). The three peptides were active at micromolar concentrations, in the order MG-H2 > MG-H1 > F5W-magainin 2. These activities differ from their hemolytic and bactericidal activities. The results demonstrate that positional hydrophobicity, which reflects the presence of short stretches of sequences rich in hydrophobic amino acids, plays an important role in the activities of leishmanicidal peptides.

The human protozoan parasite *Leishmania* is the causative agent of leishmaniasis, a disease with a broad range of clinical manifestations afflicting 12 million to 14 million people worldwide, particularly in tropical and subtropical countries (13). Chemotherapy is nowadays the only treatment available; but its efficiency is threatened by the growing incidence of resistant parasites, together with the frequent and severe side effects associated with organic pentavalent antimonials, the first-line leishmanicidal drugs (4).

Promising expectations over membrane-active antimicrobial peptides as a new class of antibiotics have been raised due to their broad spectra of activity against susceptible pathogens and the low level or lack of induction of resistance to these peptides. Their mechanisms of action are based on permeabilization of the plasma membrane of the pathogen, effected by a stoichiometric interaction with the anionic phospholipids of the outer leaflet of the membrane through a subtle equilibrium of electrostatic and hydrophobic interactions. Optimal activity and specificity are usually achieved when the positive charge and hydrophobicity are constrained within a limited range of values. A low charge causes an interaction of the peptide with the membrane that is too weak, whereas an excess charge gets the peptide stuck onto the surface, precluding its insertion. Again, for hydrophobicity, small values produce a poor interaction with the nonpolar matrix of the membrane, while values that are too high make the peptide unspecific for zwitterionic or acid phospholipid membranes. The induction of resistance requires substantial changes in the phospholipid composition of the plasma membrane, with simultaneous effects on its associated enzymatic and transport systems (for reviews, see references 23 and 32 and the references therein).

Amphibian cutaneous secretions are one of the main natural sources for antimicrobial peptides (25, 28). Some of them, such as α-helical dermaseptins (10, 12), temporins (24), and SPYY (31), have been successfully assayed with *Leishmania* promastigotes.

Magainins are antimicrobial peptides synthesized by the African clawed frog (*Xenopus laevis*) (33). Their antimicrobial activities and specificities have been extensively studied (for reviews, see references 19, 20, and 21) and are related to structural and physicochemical characteristics, for example, global characteristics, such as the aforementioned charge and hydrophobicity, plus other α-helix-specific parameters like helicity, hydrophobic moment, and hydrophobic angle (5, 6). Despite the wealth of knowledge generated in structure-activity studies of these parameters, their practical application to the design of new analogues has often failed for analogues without high degrees of similarity with the initial prototypes. Therefore, it is believed that there is a need to rely on more realistic functional parameters, i.e., those that account for local characteristics of a given region within the peptide structure rather than average global values for the entire molecule (17, 29). Positional hydrophobicity weighs the intrinsic hydrophobicity inherent to each amino acid in the sequence with its topological location within the peptide structure, thus allowing detection of the presence of hydrophobic stretches which endow the peptide with a higher affinity for membranes or which may

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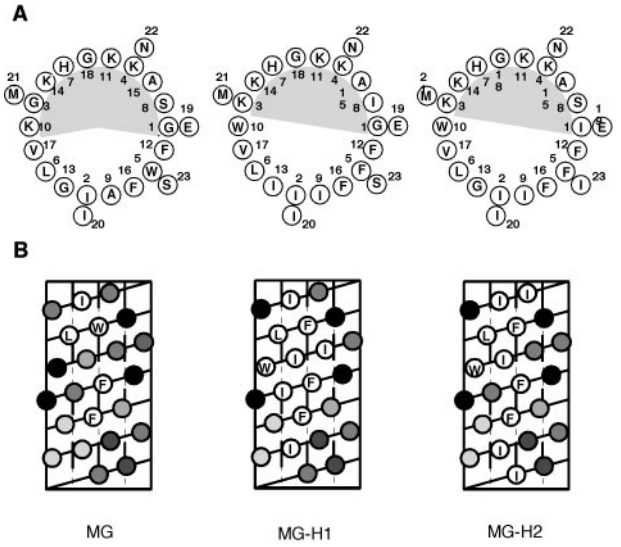

FIG. 1. F5W-magainin 2 analogues assayed for activities against *Leishmania* promastigotes. (A) Helical wheel representations. Shaded areas denote the polar area of the amphipathic helix. (B) Helical net representations, with a white-black hydrophobicity scale. Polar residues are represented in black, whereas the most hydrophobic residues are presented in white. Reprinted with permission of the American Chemical Society from reference 29.

facilitate its aggregation in solution, precluding translocation through tightly packed external barriers, such as the outer membrane in gram-negative bacteria (29).

The influence of positional hydrophobicity on the activities of antimicrobial peptides against human parasites has not yet been addressed. We have studied the leishmanicidal mechanisms of two hydrophobic magainin 2 analogues, MG-H1 (GIKKFLHIIWKFIKAFVGEIMNS) and MG-H2 (IIKKFLH SIWKFGKAFVGEIMNI), as well as their parental peptide, F5W-magainin 2 (GIGKWLHSAKKFGKAFVGEIMNS) (Fig. 1 and Table 1), against *Leishmania donovani* promastigotes. Although MG-H1 and MG-H2 are more hydrophobic than F5W-magainin 2, they share identical values for most global parameters. An exception is for the retention time in reverse-phase chromatography, which is directly linked to positional hydrophobicity. This is interpreted on the basis of the fact that in MG-H1 hydrophobic residues are grouped at the central region, promoting aggregation in water, whereas in MG-H2 they are more evenly distributed through the sequence (29). As a practical consequence, while the rate of liposome permeation was the highest for MG-H1, the antibacterial activity of MG-H1 was the lowest, given its self-aggregation state (29), which would likely hamper its ability to cross the outer membrane of *Escherichia coli* (1).

In this study, we explore the role of positional hydrophobicity in the lethal activities and mechanisms of action of these peptides against *L. donovani* promastigotes, together with the putative role of lipophosphoglycan (LPG), the primary component of the promastigote glycocalyx (15), as a defensive steric barrier for aggregated antimicrobial peptides.

MATERIALS AND METHODS

Reagents. The reagents were of the highest purity available and, unless otherwise stated, were purchased from Sigma (St. Louis, Mo.) or Fluka (Buchs, Switzerland). Fetal calf serum was obtained form Gibco-BRL (Paisley, United Kingdom). Bis(1,3-diethylthiobarbituric)trimethine oxonol (bisoxonol), SYTOX green, rhodamine 123, and D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester (DMNPE-luciferin), were obtained from Molecular Probes (Leiden, The Netherlands).

Parasites. Promastigotes of *L. donovani* strain MHOM/SD/00/1S-2D and its mutant strain R2D2, which is defective in the synthesis of the repetitive unit of LPG, were kindly provided by S. J. Turco (School of Medicine, University of Kentucky, Lexington). Parasites were grown at 25°C in RPMI 1640 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum, 24 mM $NaHCO_3$, 25 mM HEPES, 2 mM L-glutamine, 100 U of unicilin per ml, and 48 μg of gentamicin per ml at pH 7.2. For strain R2D2, 5 μg of lectin ricin agglutinin (*Ricinus communis*) per ml was added to the growth medium (16).

The 3-luc strain was derived from the parental strain described above by transfection with the 3Luc-pX63Neo expression vector to allow expression of *Photinus pyralis* luciferase mutated at its C-terminal tripeptide. This luciferase form was retained at the cytoplasm and affords real-time in vivo monitoring of changes in ATP levels (3, 18). This strain was maintained under the same conditions as the parental one, but under antibiotic pressure (50 μg of G418 per ml; Gibco-BRL).

Peptides. The peptides were synthesized by standard fluoren-9-yl methoxycarbonyl-based solid-phase methods, as described previously (22), and their purities were assessed by analytical reversed-phase high-performance liquid chromatography and ion-spray mass spectrometry. A tryptophan residue was included in each peptide to facilitate peptide quantification. This modification did not alter the structures of the peptides (22).

Leishmanicidal activity. Parasites were harvested at late exponential phase, washed twice with Hank's buffer (3) supplemented with 20 mM D-glucose (Hank's-Glc), and resuspended in this buffer at a final concentration of 2×10^7 parasites/ml. Aliquots of this suspension (120 μl) were incubated with the peptides for 4 h at 25°C and further divided into two aliquots (100 and 10 μl). The larger aliquot was assayed for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction (0.5 mg/ml, 2 h, 25°C) by solubilization with sodium dodecyl sulfate at a final concentration of 5% and read in a 450 Bio-Rad enzyme-linked immunoassay microplate reader equipped with a 600-nm filter. ED_{50} and ED_{90} were defined as the peptide concentrations that inhibited MTT reduction by control parasites by 50 and 90%, respectively, immediately after peptide incubation. Aliquots of 10 μl were transferred into a 96-well microplate, diluted 10-fold with standard growth medium devoid of phenol red, and assayed for parasite proliferation for 72 h by MTT reduction.

All assays were performed in triplicate, and the experiments were repeated at least twice. The results were normalized with respect to those for the corresponding control in the absence of the peptide.

Plasma membrane potential. Membrane potential was estimated by use of the potential-sensitive anionic dye bisoxonol, whose fluorescence increases after insertion into the membrane, once the cell becomes depolarized. Assays were performed under standard conditions with the inclusion of 0.1 μM bisoxonol in the incubation medium. Changes in fluorescence after peptide addition were monitored in a Polarstar Galaxy microplate reader (BMG Labotechnologies, Offenburg, Germany). The excitation and emission wavelengths were 544 and 584 nm, respectively. Full depolarization was defined as that obtained with the peptide CA(1-8)M(1-18) at a concentration of 2.5 μM (3).

Plasma membrane permeabilization. The protocol for plasma membrane permeabilization described by Chicharro et al. (3) was followed. Briefly, promastigotes (2×10^7 cells/ml) were incubated with SYTOX green (final concentration, 1 μM) in Hanks'-Glc for 15 min in darkness. Aliquots (100 μl) from this suspension were transferred to a 96-well microplate. After fluorescence stabilization, the peptides were added and the increase in fluorescence due to binding of the dye to intracellular DNA was measured in a Polarstar Galaxy microplate reader equipped with 485- and 520-nm filters as excitation and emission wavelengths, respectively, or by cytofluorometry in a Coulter XL EPICS cytofluorometer. Maximal parasite permeabilization was defined as that caused by 0.1% (wt/vol) Triton X-100.

Electron microscopy. Promastigotes were incubated with the peptide by the standard procedure, washed twice in Hank's buffer, fixed with 5% (wt/vol) glutaraldehyde in phosphate-buffered saline, incubated with 2.5% (wt/vol) OsO_4 for 1 h, and gradually dehydrated in ethanol (30, 50, 70, 90, and 100% [vol/vol] for 30 min each) and propylene oxide (1 h). Afterwards, they were embedded in Epon 812 resin, treated with lead citrate for contrast, and observed in a Philips 2200 electron microscope.

Modification of bioenergetic parameters. The in vivo monitoring of the changes in intracellular ATP levels was carried out as described by Luque-Ortega et al. (18). Briefly, 2×10^7 promastigotes/ml transfected with the 3Luc-pX63Neo

TABLE 1. Comparison of physicochemical parameters of the peptides used in this study with their effects on *L. donovani* promastigotes[a]

Peptide	*H* (kcal/mol)[b]	μ[b]	t_r (min)[b]	ED_{50} (μM) for hemolysis[b]	MIC[b,c] (μM)	*L. donovani* promastigotes			
						Inhibition of proliferation		Inhibition of MTT reduction	
						LD_{50} (μM)	LD_{90} (μM)	ED_{50} (μM)	ED_{90} (μM)
MG-H1	−17.7	0.596	31.5	2.9	16	2.4	4.3	5.0	20.0
MG-H2	−17.7	0.607	21.7	16	2	0.9	1.0	1.8	4.6
F5W-magainin 2	−12.6	0.488	17.6	>1,000	8	6.1	9.2	8.3	24.0

[a] Abbreviations: *H*, Hydrophobicity of the nonpolar face; μ, hydrophobic moment t_r, reverse-phase high-performance liquid chromatography retention time.
[b] Data are taken from Tachi et al. (29).
[c] MICs are those for *E. coli.*

expression vector were incubated at 25°C with the membrane-permeant luciferase substrate DMNEP-luciferin at 25 μM. When the luminescence reached a plateau, peptide was added and the ensuing decay in luminescence was monitored in a BioOrbit 1250 LKB luminometer, with readings averaged every 10 s.

The mitochondrial membrane potential ($\Delta\Psi_m$) in *L. donovani* promastigotes was measured by determination of rhodamine 123 accumulation, as described previously (9). Prior to the standard peptide assay, parasites (2×10^7 promastigotes/ml) were equilibrated with rhodamine 123 (final concentration, 0.3 μg/ml) for 5 min at 37°C, washed twice, and resuspended in Hank's-Glc. Dye incorporation was estimated in a Coulter XL EPICS cytofluorometer (excitation and emission wavelengths, 488 and 525 nm, respectively). Parasites depolarized with 7.5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were used as negative controls.

Statistical calculations. The EDs and lethal doses (LDs) were calculated by using the Litchfield and Wilcoxon algorithm with the PHARM/PCS (version 4) software package for personal computers. A paired *t* test was carried out with SIGMA-Plot (version 8.0) software.

RESULTS

Leishmanicidal activities of magainin 2 and its analogues. The three peptides (Fig. 1 and Table 1) showed leishmanicidal activities in the micromolar range, with the 90% LDs (LD_{90}s) spanning almost an order of magnitude: MG-H2 was the most active (1.0 μM), followed by MG-H1 (4.3 μM) and F5W-magainin 2 (9.2 μM). When the variations were compared with the respective LD_{50}s, MG-H2 showed the steepest variation. Accordingly, the rank for leishmanicidal activity differed from those in previous reports on positional hydrophobicity (MG-H1 > MG-H2 > F5W-magainin 2 [Table 1]) and bactericidal activity (MG-H2 > F5W-magainin > MG-H1[29]).

Inhibition of MTT reduction (EDs), measured immediately after peptide incubation, accounted for short-term effects. The EDs were consistently higher than the respective LDs. This fact, together with the fast incorporation of both peptides into the parasites (95% of the end points for both parameters were reached in less than 5 min; data not shown), provides evidence that deterioration of intracellular homeostasis proceeded progressively, with the rate highly dependent on the peptide concentration.

Role of electrostatic interactions in peptide activity. The initial electrostatic interaction between a peptide and acidic phospholipids is the main factor in the subsequent cidal specificity (20, 27). To get insight into its role in the leishmanicidal mechanism, two sets of experiments were carried out. In the first one, the ratio between the percent MTT reduction values in media with two different salinities (140 mM as a standard medium and 80 mM NaCl as a medium with low ionic strength) was determined for the three peptides at their ED_{50}s. As expected, given their identical positive charges, the ratios for the three peptides showed similar, almost twofold variations (1.8, 1.7, and 1.8 for F5W-magainin 2, MG-H2, and MG-H1, respectively).

The second set of experiments addressed the possible role of strongly anionic LPG, the main component of the promastigote glycocalyx, as a quencher of peptide activity. To this end, the peptides were assayed with mutant strain R2D2, which lacks the repetitive phosphorylated disaccharide region of LPG and, hence, is devoid of most of the anionic charge (16). The activities (ED_{50}s) of the peptides against wild-type and mutant strains were compared and were as follows (confidence intervals are given in parentheses): 7.7 μM (8.5 to 6.9 μM) and 10.4 μM (15.6 to 6.9 μM), respectively, for F5W-magainin 2; 1.6 μM (4.8 to 0.6 μM) and 1.0 μM (4.1 to 0.3 μM), respectively, for MG-H2; and 4.8 μM (8.4 to 2.8 μM) and 2.7 μM (3.6 to 2.0 μM), respectively, for MG-H1 (*P* values for all comparisons, >0.1). Thus, although LPG did modify the activities of all three peptides, the ratios of the activities against the two strains never exceeded twofold and the statistical significance was poor. From this we conclude that LPG is not an important barrier to these peptides.

Permeabilization of *L. donovani* promastigote plasma membranes. Compiled data and previous reports on these peptides together suggested that their leishmanicidal activities rely mainly on permeabilization of the promastigote plasma membrane (19). This was further evidenced by three complementary methods: plasma membrane permeabilization to SYTOX green as a vital dye, plasma membrane depolarization, and transmission electron microscopy.

Promastigote permeabilization to SYTOX green (molecular weight, ~900) is measured by the increase in its fluorescence once it becomes bound to intracellular nucleic acids, a process involving substantial damage to the plasma membrane. Figure 2 shows the concentration-dependent kinetics for the three peptides. Their activities followed the same order as that for MTT reduction (MG-H2 > MG-H1 > F5W-magainin 2). Complete permeabilization, defined as that caused by 0.1% (wt/vol) Triton X-100, was never reached, most likely because exposure of the kinetoplast DNA to the dye was not attained over the range of peptide concentrations assayed. Promastigote permeabilization was carried out in an all-or-nothing manner, as assessed by cytofluorometric analysis of dye incorporation, and illustrated that MG-H2 was the most active peptide (Fig. 3).

These results were in agreement with the dose-dependent depolarization of the plasma membrane caused by the pep-

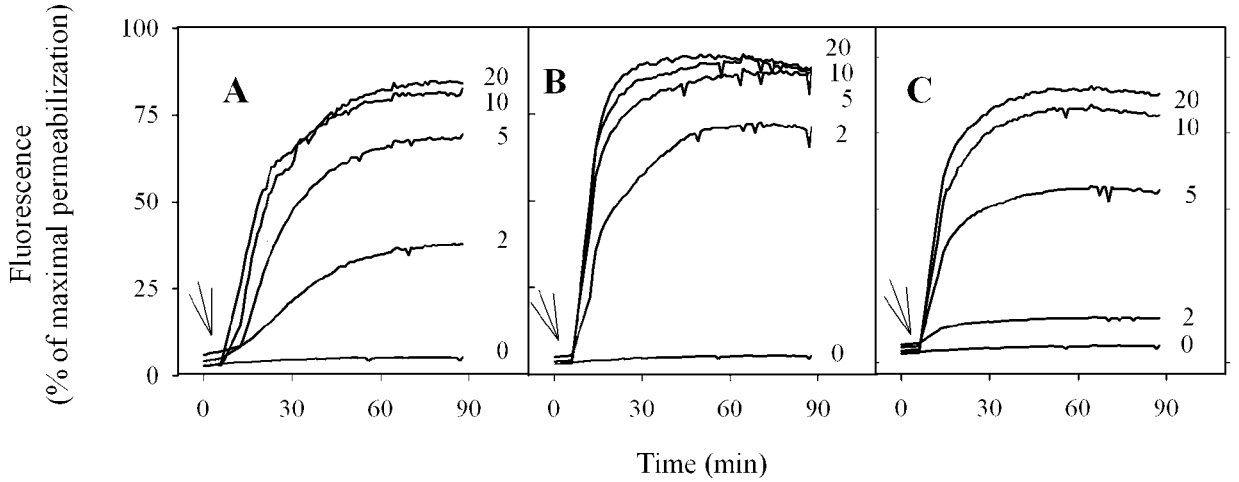

FIG. 2. Increases in SYTOX green fluorescence of *L. donovani* promastigotes caused by magainin 2 and its analogues. Parasites were incubated with 1 μM SYTOX green, as described in Materials and Methods. Once the basal fluorescence reached a constant value, the corresponding peptide was added and the increase in fluorescence (excitation wavelength, 485 nm; emission wavelength, 520 nm) was plotted as a percentage of the fluorescence relative to that of parasites fully permeabilized by the addition of 0.1% Triton X-100. (A) MG-H1; (B) MG-H2; (C) F5W-magainin 2. The times of addition of peptide are indicated by arrows. Peptide concentrations (micromolar) are shown beside their respective traces.

tides, measured by the increase in fluorescence of the anionic dye bisoxonol when it was inserted into the hydrophobic matrix of a depolarized plasma membrane. Figure 4 shows the kinetics of the process, which follows a pattern similar to that of inhibition of promastigote proliferation and SYTOX green influx. Full depolarization was defined as that obtained with 2.5 μM CA(1-8)M(1-18), a membrane-active antibiotic peptide (8).

Finally, to gain insight into the morphological damage inflicted on the promastigote by the peptides, electron microscopy was carried out with parasites treated with the peptides at their respective ED_{90}s (Fig. 5). At this concentration, all peptides produced severe damage to the plasma membrane, causing its disruption, strong intracellular vacuolization, and the observation of an electron-translucent intracellular space, suggestive of the massive release of cytoplasmic material.

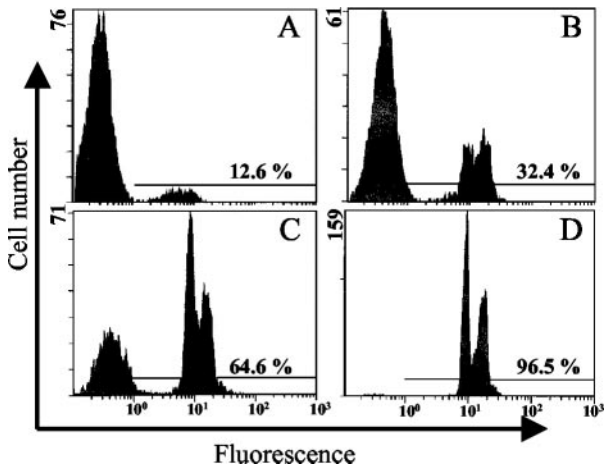

FIG. 3. Cytofluorometric analysis of SYTOX green incorporation into *L. donovani* promastigotes caused by MG-H2. Parasites were incubated under standard conditions in the presence of 1 μM SYTOX green; dye incorporation was analyzed by cytofluorometry. (A) Control; (B) 1 μM MG-H2; (C) 2.5 μM MG-H2; (D) 0.1% Triton X-100.

Decrease in intracellular ATP levels. The decay of intracellular ATP was monitored in vivo as the decrease of luminescence in luciferase-transfected promastigotes (3, 18). As depicted in Fig. 6, the three peptides caused a fast and dose-dependent decay of luminescence, with the activities of the three peptides being in the same order described above. For MG-H2, the decay in fluorescence was more pronounced and was observable even at 1 μM, with a slight and transient increase at short observation times.

Variation of $\Delta\Psi_m$. The reported rapid exhaustion of ATP caused by the peptides might be due to inhibition of oxidative phosphorylation, the essential ATP source in *Leishmania* promastigotes (30). To discard this alternative, variation of $\Delta\Psi_m$ after peptide addition was monitored by rhodamine 123 accumulation. The levels of accumulation of the three peptides were consistently maintained, even at peptide concentrations higher than the ED_{90}s. Partial mitochondrial depolarization was observed only for F5W-magainin 2, but the level of depolarization was much lower than that caused by the protonophore FCCP (Fig. 7). Beyond 1 h after peptide addition, $\Delta\Psi_m$ underwent a steady decline, likely due to the progressive deterioration of intracellular homeostasis after peptide permeabilization, and thus was more a consequence of than an essential step in the killing mechanism.

DISCUSSION

Despite the extensive documentation on the antimicrobial activities of magainins (19) and their dependence on structural parameters (5, 6), their roles as leishmanicidal agents have not been sufficiently explored, whereas their activities against other human protozoan pathogens such as *Acanthamoeba* (26), *Cryptosporidium* (11), and even other trypanosomatids such as *Trypanosoma cruzi* (14) have been studied previously.

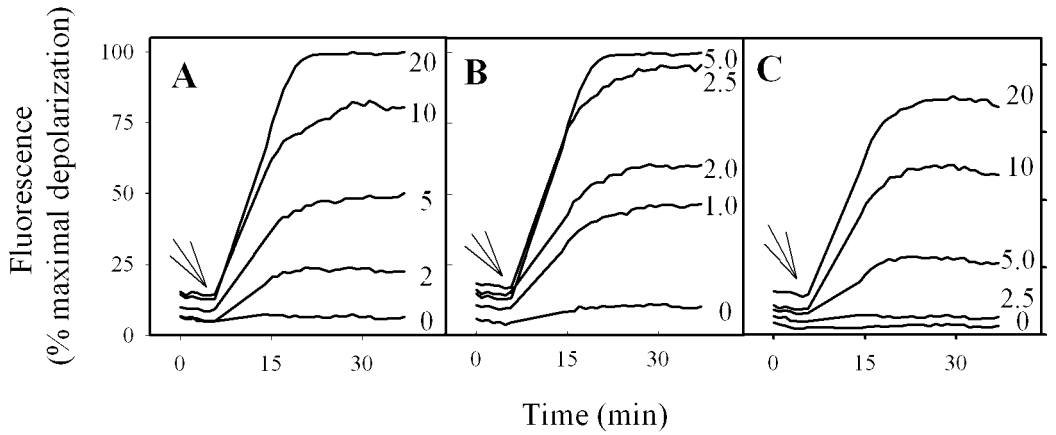

FIG. 4. Depolarization of the plasma membranes of *L. donovani* promastigotes by F5W-magainin and its analogs. Variations in plasma membrane potential were monitored by measurement of the changes in the fluorescence of the potential-dependent dye bisoxonol (excitation wavelength, 540 nm; emission wavelength, 580 nm). The times of peptide addition are indicated by arrows and are plotted as the percentage of the maximal depolarization obtained by incubation with 2.5 μM CA(1-8)M(1-18). (A) MG-H1; (B) MG-H2; (C) F5W-magainin 2. Peptide concentrations (micromolar) are shown beside their respective traces.

Aside from the potential clinical interest in the development of new leishmanicidal peptides, the plasma membrane of *Leishmania* promastigotes is unusual relative to those of standard eukaryotes: (i) it is extremely rich in glycosylphosphatidylinositol-anchored molecules such as leishmanolysin, a metalloproteinase with a broad substrate specificity, and LPG, the main component of the anionic glycocalyx that surrounds the parasite; and (ii) the replacement of a damaged membrane by endo- and exocytosis is constrained to the flagellar pocket, which is the sole area devoid of subpellicular microtubules (for a recent review, see reference 15).

F5W-magainin 2 and its two analogues killed *Leishmania* promastigotes by plasma membrane permeabilization. After the addition of the peptides at their respective LD_{90}s, the parasites were quickly and dramatically depolarized, with the extracellular influx of otherwise membrane-impermeant dyes, severe disruption of the plasma membrane, and fast bioenergetic collapse, similar to the activities of other leishmanicidal membrane-seeking peptides, such as cecropin A-melittin hybrids (3, 9, 18) and dermaseptins (12).

Interestingly, the percentage of SYTOX green accumulation at a given peptide concentration was generally higher than that needed for depolarization; this was remarkably noticeable for MG-H2 and, to a lesser extent, for F5W-magainin 2. While the influx of SYTOX green (molecular weight, ~900) is cumulative and almost irreversible, once the dye becomes bound to nucleic acids, depolarization requires less membrane damage and is a dynamic, reversible process that depends on ionic gradients across the plasma membrane. Hence, the membrane potential after peptide addition is the outcome of two opposite processes: dissipation through ion leakage caused by peptide-driven membrane permeation and recovery by ATP-dependent pumps, which end up exhausting intracellular ATP. This duality is especially noticeable for MG-H2 and is noticeable to a

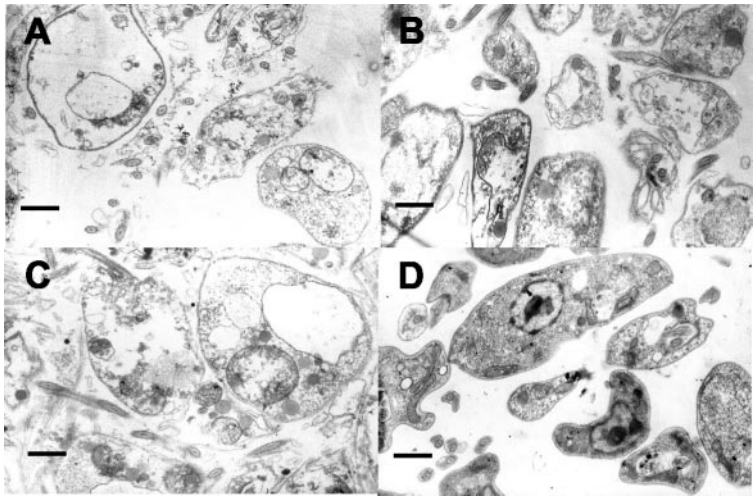

FIG. 5. Electron microscopy of *L. donovani* promastigotes treated with F5W-magainin 2 and its analogues. Parasites were incubated for 4 h with the corresponding peptide at its LD_{90}. (A) 5 μM MG-H1; (B) 1 μM MG-H2; (C) 10 μM F5W-magainin 2; (D) control parasites.

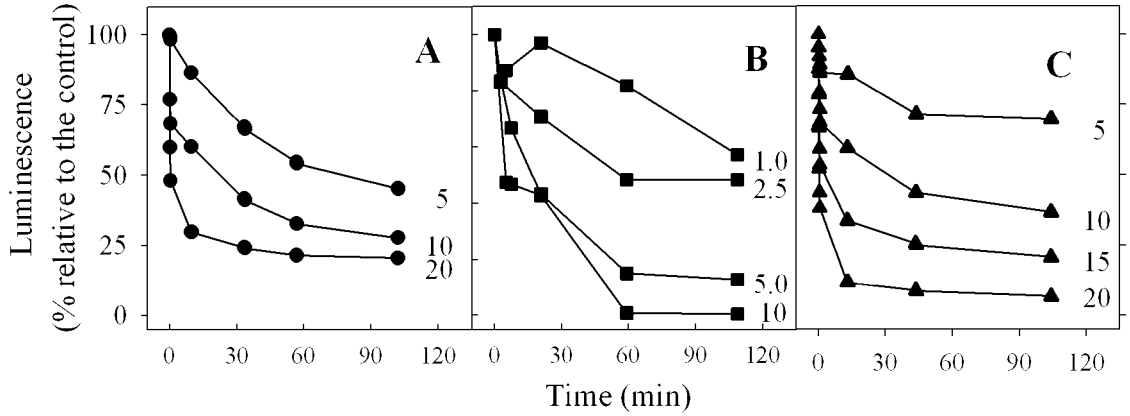

FIG. 6. In vivo monitoring of changes in intracellular ATP levels in *L. donovani* promastigotes after addition of F5W-magainin 2 and analogues. *L. donovani* promastigotes from the 3-luc strain transfected with a cytoplasmic form of firefly luciferase were loaded with 25 μM DMNPE-luciferin as the membrane-permeant substrate. Once constant luminescence was attained, the peptides were added (time zero) and the decrease in luminescence was monitored. The peptide concentrations (in micromolar) are indicated at the sides of the respective traces. (A) MG-H1; (B) MG-H2; (C) F5W-magainin 2.

lesser extent for F5W-magainin 2 at lower concentrations: both peptides cause membrane permeabilization by the transient formation of a mixed peptide-phospholipid pore (wormhole model) (20), once the peptide concentration on the outer membrane leaflet exceeds a threshold. Beneath this point, peptides may still cause transient perturbation of the membrane, with ion leakage through a carpet-like model (27). This may account for the partial depolarization observed with MG-H1, which causes membrane permeabilization in a detergent-like manner (29) and to which the percentages of both SYTOX green and bisoxonol accumulation were more similar (29). The all-or-nothing permeabilization of promastigotes observed for MG-H2 reinforces this hypothesis.

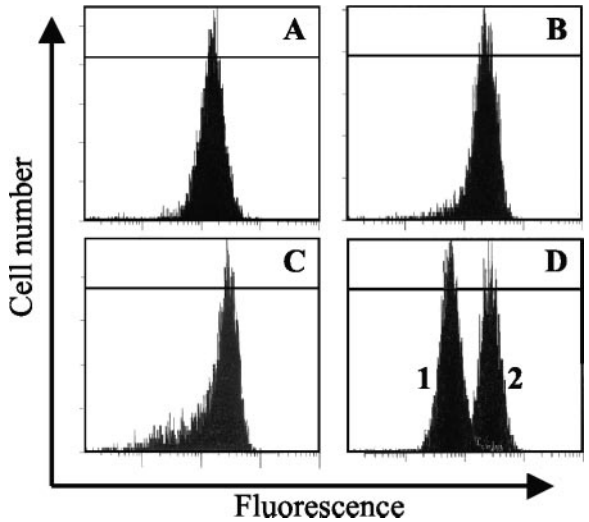

FIG. 7. Variations in $\Delta\Psi_m$ values for *L. donovani* promastigotes assessed by determination of the levels of accumulation of rhodamine 123 fluorescence. Parasites were loaded with rhodamine 123 as described in Materials and Methods, and dye accumulation was measured by cytofluorometry. (A) 10 μM MG-H1; (B) 2.5 μM MG-H2; (C) 20 μM F5W-magainin 2; (D) parasites treated with 7.5 μM FCCP (peak 1) and control parasites (peak 2).

One of the consequences of the wormhole model is that after the spontaneous disruption of the pore, peptides are distributed on both sides of the membrane (20), with those facing the cytoplasm gaining access to potential intracellular targets (20). Two of our findings agree with this scenario: the partial mitochondrial depolarization observed with F5W-magainin 2 and the consistently lower EDs than LDs of a given peptide. The first of these findings, the in vivo depolarization of mitochondria, has been observed in hamster spermatozoa incubated with magainin (7). Although we cannot rule out the existence of intracellular targets for these peptides in *Leishmania*, the extent of mitochondrial depolarization is much lower than that obtained with FCCP, a typical protonophore. As to the second finding, EDs represent short-term effects, such as membrane permeabilization, while LDs represent the final outcome, i.e., the loss of intracellular homeostasis that can be delayed by the aforementioned repair mechanisms. This differentiation between both processes is blurred or even absent for peptides with stronger, detergent-like permeabilization effects, such as the cecropin A-melittin hybrids (3, 9).

Positional hydrophobicity accounts for the presence of hydrophobic patches within a structure. According to this criterion, MG-H1 and MG-H2 differ widely, although their average hydrophobicities, as well as other parameters, are practically identical. Positional hydrophobicity promotes peptide aggregation in solution, which, in turn, produces high local concentrations of the peptide inserted in the membrane, and these concentrations tend to improve permeabilization. On the other hand, a larger size—relative to those of monomers—impairs aggregate transport through biological barriers (1, 17, 29). These facts should allow differentiation of the effects of highly aggregating peptides on cells with relatively loose glycocalyxes, such as erythrocytes, from those on targets with robust, highly impermeable barriers, of which the outer membrane of gram-negative bacteria would be a paradigm. The rank order for

leishmanicidal activity (MG-H2 > MG-H1 > F5W-magainin 2) differed from those for both hemolytic activity (MG-H1 > MG-H2 > F5W-magainin 2) and bactericidal activity (MG-H2 > F5W-magainin 2 > MG-H1) against *E. coli* (29). Although one might conclude that this is due to the characteristics of the glycocalyx of the *Leishmania* promastigote, in which LPG covers at least 40% of the surface and precludes lysis by complement and attack by sandfly or macrophage hydrolases (15), the fact that the same ranking is observed for strain R2D2, which has a truncated LPG, makes the hypothesis questionable. Although LPG might seem to provide partial protection against MG-H1 and MG-H2 analogues, in contrast to F5W-magainin 2, the relevance of this finding is statistically questionable; and the level of protection attained with LPG against these two peptides never attained the levels found against more cationic peptides such as indolicidin (2) and cecropin A-melittin hybrids (8).

Our work has extended the antimicrobial spectrum of magainin and its two analogues to *Leishmania* promastigotes. Furthermore, the fact that the cidal activities of the two analogues with contrasting positional hydrophobicity patterns differed more than fourfold, despite similar or identical values for most parameters based on average structure, reinforces positional hydrophobicity as a crucial tool for the fine-tuning of activity modulation and points toward its implementation in the design of new and improved leishmanicidal peptides.

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